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Haemopoiesis in the beagle foetus after *in utero* irradiation

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20. ABSTRACT (continued)

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Haemopoiesis in the beagle foetus after *in utero* irradiation

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On day 33 of gestation, foetal beagles were irradiated *in utero* (0·9 Gy of ^{60}Co γ-irradiation, 0·4 Gy/min). Foetal haematocytopoiesis was studied during the third trimester of gestation (days 42–55). Peripheral blood nucleated cell counts were 33 per cent lower than normal on day 44 and continued to be lower until day 49, when values became higher than normal. Splenic cellularities of irradiated pups on day 44 were more than 3 times those of the nonirradiated, but thereafter they were similar to normal. Differences in haemopoietic progenitor cell activity between irradiated and normal foetuses were observed. In comparison with the other foetal tissues, the foetal liver appeared to experience greater radiation injury. For example, on day 44, the irradiated liver BFU-E, CFU-E, and GM-CFC per 10^5 cells were almost fivefold lower than normal values. Spleens of irradiated foetal beagles contained a marked increase in all haemopoietic progenitor cells (BFU-E, CFU-E, and GM-CFC) and recognizable proliferative granulocytic cells and nucleated erythroid cells. The haemopoietic activity of the irradiated bone marrow during days 42–44 was similar to that of the irradiated spleen, and compensated for the damaged liver. However, unlike the irradiated spleen, the irradiated bone marrow had decreased BFU-E activity compared with the values for the nonirradiated bone marrow during days 48–55. Until day 50, the irradiated marrow contained fewer recognizable proliferative granulocytic cells but more nucleated erythroid cells.

Indexing terms: foetal haemopoiesis, beagle, irradiation.

1. Introduction

Neurological and skeletal abnormalities in prenatally irradiated juveniles and young adults have been documented in animal model experimentation (Brent 1971, Rugh 1971, Sikov and Muhlum 1969), human epidemiologic reports (Gaulden and Murry 1980, National Council on Radiation Protection and Measurements 1977, Upton 1970) and accounts of Hiroshima and Nagasaki atomic bomb survivors (Committee for the Compilation of Materials on Damage Caused by the Atomic Bombs in Hiroshima and Nagasaki 1981). Due to a biasing of factors while collecting information, there is a pronounced dichotomy in the literature concerning the lower incidence of myeloproliferative anomalies following *in utero* or preconception exposure to diagnostic X-rays and the higher incidence following the atomic bombings of Hiroshima and Nagasaki (Oppenheim *et al.* 1974).

Our laboratory has established two animal models to investigate the long-lasting myeloproliferative effects of *in utero* irradiation during mid-gestation. The haemopoietic perturbations in B6D2F1 mice exposed during mid-gestation (day 10·5) to various doses of gamma radiation (0·5–3·0 Gy ^{60}Co, 0·4 Gy/min) have recently been reported (Weinberg 1983, Weinberg and MacVittie 1982, Weinberg *et al.* 1981). These prenatally irradiated mice were studied at four selected ages: (*a*) foetus at day 14·5 of gestation, (*b*) 9-day-old neonate, (*c*) 15-day-old juvenile, and (*d*) 13-week-old young adult.

The dog was used in the second model because of its larger size and the abundance of information already available on normal haematopathology (Andersen and Good 1970). Data was collected from the foetuses and from the pregnant animal throughout the study.

This paper delineates foetal beagle haematocytopoiesis during the third trimester of gestation (days 42–59) after *in utero* exposure to 0·9 Gy ^{60}Co (dose rate of 0·4 Gy/min) during mid-gestation, day 33 (equivalent to day 10·5 in mouse foetal development). Differences between irradiated and nonirradiated groups included (*a*) fluctuations in peripheral blood total nucleated counts, (*b*) changes in spleen size and haemocytopoietic activity, (*c*) decreased liver erythrocytopoiesis, and (*d*) increased bone marrow haemocytopoietic activity.

2. Materials and methods

Pregnant beagles with confirmed dates of conception were obtained from Hazelton Research Animals (Cumberland, VA) and the Springville Laboratory of Roswell Park Memorial Institute (Buffalo, NY). Dogs were housed individually in stainless-steel cages in temperature-controlled rooms with a 12-hour light–dark cycle. They were fed a diet of kibbled laboratory dog food (Respond 1600, Country Food Division of Agway Inc., Syracuse, New York) and water *ad libitum*, supplemented once a week with a high-protein canned-meat ration. All dogs received continuous attention from the staff of the Institute's Veterinary Department.

Dog phantoms were used to obtain dosimetry data in determining the midline body dose of 0·9 Gy to the pregnant dog. At 33 days of pregnancy (gestation period of beagle is about 60 days), dogs were placed in a plastic holding cage. They received a midline radiation exposure of 0·9 Gy from bilaterally positioned ^{60}Co elements containing 5·18 PB_q (140 000 Ci), at a dose rate of 0·4 Gy/min. This dose has been found to have significant effects on the pregnant dog haemopoiesis (Weinberg *et al.* 1983). Nonirradiated foetal beagles served as the control group.

At selected ages of gestation (days 42–55), pregnant dogs were anaesthetized with Surital (thiamylal sodium from Parke Davis, Detroit, MI) and maintained during surgery with halothane–oxygen–nitrous oxide. A ventral midline incision was made on each occasion, and one horn of the uterus with foetal pups was removed. Each dog was allowed to recover from surgery for at least 5 days before the removal of the other horn of uterus with the remainder of the pups from the litter. Each pup was dissected free from maternal connective tissues. The umbilical cord was clamped and immediately ligated. Each foetal pup was washed in a sterile physiologic saline solution and then swabbed with 70 per cent ethanol. Samples of heparinized blood were obtained by cardiac puncture for study of numbers of total nucleated cell counts per mm^3.

The spleen, a section of liver, both femora, tibiae, and humeri were removed from each pup and washed twice in cold sterile saline. The liver and spleen were minced into small pieces in Supplemented Alpha Medium (SAM) + 2 per cent foetal calf serum (FCS) (Weinberg *et al.* 1981). With the aid of a 5 ml pipette and gentle aspiration, a single-cell suspension was prepared of each tissue pooled from the pups. Similarly, the marrow of pups was flushed out of the long bones with SAM + 2 per cent FCS and pooled. A single-cell suspension was prepared by gentle aspiration through a 23-gauge needle fitted onto a 3 c.c. hypodermic syringe.

Cytospin smears prepared for each tissue cell suspension were stained with benzidine and counterstained with Wright's-Giemsa blood stain solutions for evaluation of the different haemopoietic cellular elements.

Nonirradiated and irradiated foetal bone marrow, spleen, and liver cells were cultured ($2–5 \times 10^5$ nucleated cells of each tissue per ml were plated) in soft agar cultures (MacVittie and Walker 1978, 1980) with 15 per cent (v/v) plasma from endotoxin-stimulated dogs, which served as the source of colony-stimulating activity (CSA). After 10 days of incubation, colonies of 50 cells or more were counted and considered to be derived from the granulocyte-macrophage colony-forming cell, GM-CFC. Microplasma clot cultures were established (Weinberg *et al.* 1981) with anaemic sheep plasma (Step III, Connaught Labs, Swiftwater, PA, lot no. 3023-3, 6·7 units per mg protein) as the source of erythropoietin (EPO). Cultures ($2·5 \times 10^4$ nucleated liver or spleen cells per 0·1 ml, $5·0 \times 10^4$ nucleated marrow cells per 0·1 ml) with 0·05 units EPO per 0·1 ml were harvested after 72 hours of incubation for the erythroid colony-forming unit (CFU-E). Colonies of eight or more benzidene-stained positive cells were counted as representing the more mature erythroid progenitor cell, CFU-E. Clots plated with 0·3 units EPO per 0·1 ml were harvested at 9 days of culture for the erythroid burst-forming unit (BFU-E). Clusters of benzidine-stained positive cells or large colonies with more than 50 benzidine-stained positive cells were counted as the younger erythroid progenitor cell, BFU-E.

At least three pups were used for each day of gestation studied (days 42, 43, 44, 47, 48, 49, 50, 52, 53, 54, and 55) and each time point studied was repeated at least two times.

3. Results

3.1. *Peripheral blood*

Foetal peripheral blood nucleated cell counts are shown in figure 1. Day-43 (10 days after irradiation) irradiated foetal blood cell counts were 33 per cent lower than normal counts. These values remained lower than those of normal until day 49, at which time counts increased above normal values and continued to be higher throughout the duration of the study (to day 55 of gestation).

3.2. *Haemopoietic progenitor cells*

3.2.1. *Erythroid burst-forming unit*

The normal canine foetal liver was the primary site of erythrocytic activity during the early stages of gestation and continued as the predominant contributor of BFU-E until day 46, when there was a change in the erythrocytic locus to the foetal spleen. Irradiated foetal liver values were dramatically affected by irradiation on day 33 (table 1). Day-42 irradiated liver CFU-E were significantly lower ($p < 0·005$) than

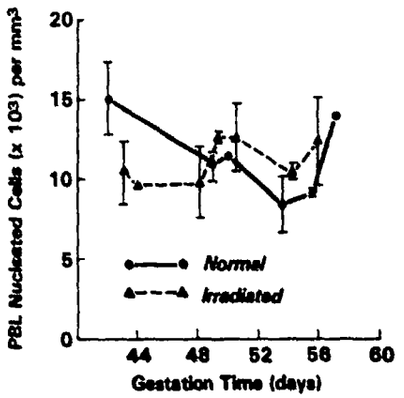

Figure 1. Foetal beagle peripheral blood total nucleated cell counts per mm^3. Each point at a given time represents the mean ± SEM of normal and irradiated foetal beagles (irradiated on day 33 of gestation).

	Liver		Spleen		Bone marrow	
Age	Normal	Irradiated	Normal	Irradiated	Normal	Irradiated
Days 42–44 (19:10)	29·6	6·7*	9·5	19·8*	1·5	5·3*
Days 48–50 (18:17)	7·8	13·5	23·0	28·0	6·1	4·6
Days 52–55 (16:7)	11·6	2·3*	27·0	69·0*	18·5	12·4*

* Statistically significant difference ($p < 0.05$).

Table 1. Erythroid burst-forming unit (BFU-E) values in foetal beagle tissues. Values are expressed as mean BFU-E per 10^5 cells. Numbers in parentheses are the numbers of pups studied during each gestation period investigated (normal pups : irradiated pups).

normal, but within normal range by day 48. Irradiated spleen BFU-E were above normal throughout the study (days 42–44 and days 52–55, $p < 0.05$). Normal bone marrow erythropoietic activity was evident at day 42. Subsequent maturation of both groups of bone marrow accounted for a secondary contributor of BFU-E during days 50–55. Commencing with day 42, bone marrow data reflected the same extent of radiation injury as the foetal liver and the foetal spleen (days 42–44 and days 52–55, $p < 0.05$).

3.2.2. *Erythroid colony-forming unit*

On both nonirradiated and irradiated foetuses, the more mature erythroid progenitor cell (CFU-E) appeared to follow a sequence of incidence similar to that of its precursor cell (table 2). The nonirradiated foetal liver contained the highest concentration of CFU-E from days 42–50. At this time, the foetal liver experienced a precipitous decline in CFU-E activity, and was replaced by the foetal spleen as the primary erythrocytopoietic locus. The decrease on days 42–46 in irradiated liver

Age	Liver		Spleen		Bone marrow	
	Normal	Irradiated	Normal	Irradiated	Normal	Irradiated
Days 42–44 (19:10)	882	159*	76	81	20	70*
Days 48–50 (18:17)	270	249	135	192	110	81
Days 52–55 (16:7)	97	80	330	651*	69	162*

* Statistically significant difference ($p < 0.05$).

Table 2. Erythroid colony-forming unit (CFU-E) values in foetal beagle tissues. Values are expressed as mean CFU-E per 10^5 cells. Numbers in parentheses are the numbers of pups studied during each gestation period investigated (normal pups : irradiated pups).

BFU-E (table 1) following exposure on day 33 resulted in a significant reduction of liver CFU-E (days 42–44, $p < 0.02$) during the course of the study. Related to BFU-E per 10^5 cells during foetal development (days 42–55), normal foetal marrow CFU-E per 10^5 cells was considerably less than values of foetal liver or foetal spleen, Mid-gestation irradiation resulted in elevated spleen CFU-E activity and marrow CFU-E activity (table 2).

3.2.3. *Granulocyte-macrophage colony-forming cell*

The presence of considerable numbers of GM-CFC per 10^5 nucleated cells cultured was detected in the normal foetal bone marrow, spleen, and liver at day 42 of gestation (table 3). Thereafter the bone marrow persisted as the predominant site of granulocytic progenitor cell production, while activity in the foetal liver and spleen gradually declined. Mid-gestation irradiation had the most pronounced effect on liver GM-CFC activity (table 3). Irradiated liver values by day 52 were 65 per cent lower ($p < 0.05$) than those for nonirradiated foetuses. Both irradiated spleen and

Age	Liver		Spleen		Bone marrow	
	Normal	Irradiated	Normal	Irradiated	Normal	Irradiated
Days 42–44 (19:10)	37	25	76	119	111	163
Days 48–50 (18:17)	24	36*	57	124*	127	291*
Days 52–55 (16:7)	26	9*	77	73	230	133*

* Statistically significant difference ($p < 0.05$).

Table 3. Granulocyte–macrophage colony-forming cell (GM-CFC) values in foetal beagle tissues. Values are expressed as mean GM-CFC per 10^5 cells. Numbers in parentheses are the numbers of pups studied during each gestation period investigated (normal pups : irradiated pups).

bone marrow exhibited increased GM-CFC production during days 42–50 (i.e., >46 per cent increase for days 42–44 and >117 per cent increase for days 48–50, $p<0.02$).

3.2.4. *Cellular morphology*

The haemopoietic cellular differential distributions of nonirradiated and irradiated foetal liver, spleen, and bone marrow during days 42–55 of foetal gestation are shown in tables 4, 5, and 6, respectively. The highest percentage of nucleated erythroid cells was observed in the normal foetal liver (table 4). Mid-gestation irradiation resulted in slightly fewer foetal liver nucleated erythroid cells during days 44–48 than normal, reflecting the decrease in BFU-E and CFU-E activity noted above. Normal foetal spleen appeared to be a secondary source of nucleated erythroid cells during haematopoietic development (table 5). The per cent nucleated erythroid elements of irradiated spleens were increased above normal values (days 44–50), similar to the increases observed with irradiated spleen BFU-E and CFU-E data.

Granulocytic cellular elements were most prominent in the normal foetal marrow on day 42, and continued throughout the third trimester of gestation (table 6). Proliferative and mature granulocytic cells were less prominent in the normal foetal liver and foetal spleen. The outstanding decrease in irradiated marrow granulocytic cells (table 6) appears to be at the expense of nucleated erythroid cells. Lymphocytes were equally evident in the normal foetal spleen and foetal bone marrow, but they were relatively few in the foetal liver.

3.2.5. *Spleen cellularity*

Seven days after irradiation (day 40 of gestation), foetal spleen cellularity was 76·8 per cent higher than normal values (figure 2). Thereafter (days 46–55) all irradiated values were within normal range.

	Granulocyte				Nucleated erythroid		Lymphocytes	
	Proliferative†		Mature‡					
Age	Normal	Irradiated§	Normal	Irradiated	Normal	Irradiated	Normal	Irradiated
Day 42	1·0		5·0		83·0		8·5	
Day 44	0·0	2·5	1·0	3·1	89·0	80·7	9·0	5·4
Day 48	1·0	1·4	2·0	6·4	82·3	70·7	10·0	9·9
Day 49	1·0		11·0		69·0		11·0	
Day 50	1·0	0·5	1·0	3·4	87·0	92·2	10·0	2·4
Day 52	4·0		4·3		74·0		12·0	
Day 54	2·0	2·3	17·0	8·5	65·0	66·4	8·0	12·9
Day 55	0·7		4·0		63·5		15·5	

† Proliferative granulocytic cells include myeloblast, promyelocyte, and myelocyte.
‡ Mature granulocytic cells include metamyelocyte and mature granulocyte.
§ Foetal beagles were irradiated *in utero* on day 33 of gestation.

Table 4. Differential distribution of haemopoietic cells in beagle foetal liver. Values are expressed as mean per cent for pooled foetal liver cells on cytospin smears. Each time point represents at least two groups of pups. Differences from 100 per cent are made up by plasma cells, megakaryocytes and monocytes

	Granulocyte				Nucleated erythroid		Lymphocytes	
	Proliferative†		Mature‡					
Age	Normal	Irradiated§	Normal	Irradiated	Normal	Irradiated	Normal	Irradiated
Day 42	2·0		0·0		38·0		59·0	
Day 44	26·0	11·6	9·0	11·2	27·0	39·0	36·0	33·4
Day 48	14·0	20·7	3·0	3·6	32·5	39·5	49·5	51·4
Day 49	5·0		3·5		40·0		46·0	
Day 50	4·0	6·5	2·0	5·6	33·0	40·2	58·0	47·6
Day 52	0·5		0·5		51·0		45·5	
Day 54	0·0	2·8	7·0	4·8	44·0	43·3	47·0	44·3
Day 55	5·3		1·7		22·7		62·7	

† Proliferative granulocytic cells include myeloblast, promyelocyte, and myelocyte.
‡ Mature granulocytic cells include metamyelocyte and mature granulocyte.
§ Foetal beagles were irradiated *in utero* on day 33 of gestation.

Table 5. Differential distribution of haemopoietic cells in beagle foetal spleen. Values are expressed as mean percent for pooled foetal spleen cells on cytospin smears. Each time point represents at least two groups of pups. Differences from 100% are made up by plasma cells, megakaryocytes and monocytes.

	Granulocyte				Nucleated erythroid		Lymphocytes	
	Proliferative†		Mature‡					
Age	Normal	Irradiated§	Normal	Irradiated	Normal	Irradiated	Normal	Irradiated
Day 42	14·0		25·0		6·0		51·0	
Day 44	43·0	7·3	38·0	55·8	3·0	16·3	10·0	17·2
Day 48	25·0	10·6	45·5	36·8	4·7	16·6	28·0	33·5
Day 49	3·0		21·0		7·0		68·0	
Day 50	10·0	7·2	23·0	1·8	10·0	87·8	56·0	57·3
Day 52	16·4		24·3		9·3		47·7	
Day 54	1·0	14·1	18·0	31·9	21·0	9·5	58·0	45·9
Day 55	8·0		23·3		7·7		55·0	

† Proliferative granulocytic cells include myeloblast, promyelocyte, and myelocyte.
‡ Mature granulocytic cells include metamyelocyte and mature granulocyte.
§ Foetal beagles were irradiated *in utero* on day 33 of gestation.

Table 6. Differential distribution of haemopoietic cells in the beagle foetal bone marrow. Values are expressed as mean percent for pooled foetal bone marrow cells on cytospin smears. Each time point represents at least two groups of pups. Differences from 100% are made up by plasma cells, megakaryocytes, and monocytes.

4. Discussion

This paper presents an overview of the development of blood cell production in the normal and irradiated (mid-gestation on day 33) beagle foetus during the last trimester of gestation (days 42–55). It is in this phase of gestation that the canine foetus is rapidly growing and the haemopoietic organs are developing their roles in

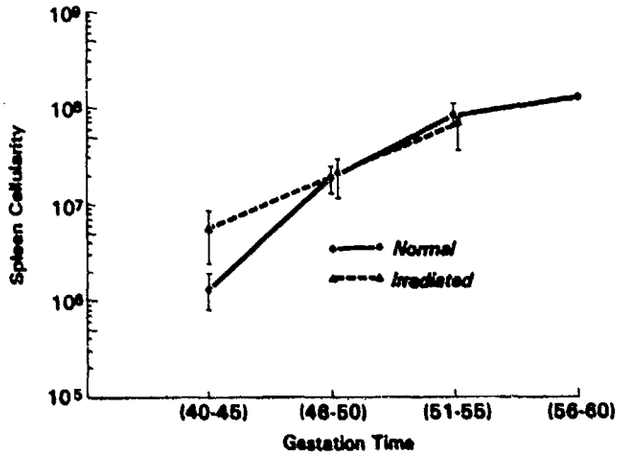

Figure 2. Spleen cellularity of normal (days 40–60) and irradiated (days 40–55) foetal beagles (irradiated on day 33 of gestation). Each point represents the mean ± SEM value.

the regulation of the adult haematopoietic system. *In vitro* clonogenic assays and morphological examination of cell suspensions from the foetal blood cell-forming tissues revealed the sequence of development of granulocytopoiesis and erythrocytopoiesis in the normal and irradiated foetal beagles.

Nothdurft *et al.* (1981) recently reported data that concur with ours, that the bone marrow is the predominant site for granulocytopoiesis during the third stage of foetal development and that the spleen accounts for myelopoietic activity, but to a lesser degree. However, their studies with six different foetal beagle tissues monitored only the granulocyte-macrophage progenitor cell.

Irradiation of the foetal beagle *in utero* at mid-gestation resulted in profound changes in foetal liver and spleen haematocytopoiesis. Haemopoietic progenitor cell production (BFU-E, CFU-E, and GM-CFC) in the irradiated foetal liver was reduced. The irradiated foetal spleen appeared to compensate for the reduced hepatic activity. This was suggested by (*a*) a 76 per cent increase in spleen cellularity, between day 40 and 45, (*b*) an increase in spleen BFU-E, CFU-E, and GM-CFC production, and (*c*) an increase in nucleated erythroid cells. Similarly, irradiated bone marrow erythrocytopoietic and granulocytopoietic activity increased to above normal values.

The observed perturbations in peripheral blood cell counts, liver, spleen, and bone marrow haemocytopoietic activity, may be a result of radiation damage to the migrating stem cells during the stage of active organogenesis (day 33) (Johnson and Moore 1975, Metcalf and Moore 1971, Yoffey 1971), or they may be due to random cell damage that is of a sufficient degree throughout the blood cell-forming tissues to result in defects in cell-to-cell interactions in the developing haematopoietic microenvironments regulating haemocytopoiesis (Mole 1982).

Stützel *et al.* (1982) have recently described the higher incidence of myeloproliferative disorders in dogs that had been irradiated *in utero*. However, their studies differ from ours because their dogs were continuously exposed to ^{60}Co (1 R per day) beginning at day 21 of gestation, and ours received a single exposure of 0·9 Gy ^{60}Co at 0·4 Gy min.

The data accumulated on the incidence of myeloproliferative malignancies (i.e., frequency and age of onset of leukaemia) after the bombings of Hiroshima and Nagasaki (Anderson and Yamamoto 1970, Committee for the Compilation of Materials on Damage Caused by the Atomic Bombs in Hiroshima and Nagasaki 1981, Mole 1982) contradict the available information from clinical centres that treat pregnant women with radiation therapy (Oppenheim *et al.* 1974), where the incidences are low. The data presented in this paper adds further evidence that myeloproliferative perturbations do occur in the beagle foetus during the third trimester of development following a single exposure at mid-gestation to 0·9 Gy gamma irradiation.

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